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Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF

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Abstract

The quantification of illicit drug and pharmaceutical residues in sewage has been shown to be a valuable tool that complements existing approaches in monitoring the patterns and trends of drug use. The present work delineates the development of a novel analytical tool and dynamic workflow for the analysis of a wide range of substances in sewage-based samples. The validated method can simultaneously quantify 51 target psychoactive substances and pharmaceuticals in sewage-based samples using an off-line automated solid phase extraction (SPE-DEX) method, using Oasis HLB disks, followed by ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOF) in MS^e. Quantification and matrix effect corrections were overcome with the use of 25 isotopic labeled internal standards (ILIS). Recoveries were generally greater than 60% and the limits of quantification were in the low nanogram-per-liter range (0.4-187 ng L^{-1}). The emergence of new psychoactive substances (NPS) on the drug scene poses a specific analytical challenge since their market is highly dynamic with new compounds continuously entering the market. Suspect screening using high-resolution mass spectrometry (HRMS) simultaneously allowed the unequivocal identification of NPS based on a mass accuracy criteria of 5 ppm (of the molecular ion and at least two fragments) and retention time (2.5 % tolerance) using the UNIFI screening platform. Applying MS^e data against a suspect screening database of over 1,000 drugs and metabolites, this method becomes a broad and reliable tool to detect and confirm NPS occurrence. This was demonstrated through the HRMS analysis of three different sewage-based sample types; influent wastewater, passive sampler extracts and pooled urine samples resulting in the concurrent quantification of known psychoactive substances and the identification of NPS and pharmaceuticals.

Keywords

High resolution mass spectrometry, Sewage-base epidemiology, Multi-residue method, Dynamic workflow, Screening, Psychoactive drugs

1. Introduction

The illicit use of psychoactive substances is a worldwide phenomenon with incalculable consequences on society. Estimates from 2012, indicate that between the 4 and 7 % of the world´s population has used an illicit substance at least once during the previous year [1]. Even though the overall European drug situation is generally stable, a graduated and complex situation is emerging [2]. Classic drugs, such as heroin, cocaine and cannabis remain dominant but the drug market is now peppered with a vast array of synthetic psychoactive substances. The EU Early Warning System for first time notified the discovery of 101 new psychoactive substances (NPS) in 2014 [3]. Illicit drug use and prevalence estimations are typically based on population surveys, clinical cases, seizures and mortality rates related to use. In addition to known problems with bias and a lack of representativeness, these approaches also require a long time to establish a general overview of the illicit drug situation and, generally, results are published several years post survey [4].

Pharmaceuticals arising from human, veterinary and aquaculture use, occur in the aquatic environment as one of the largest sources of contaminants of anthropogenic origin and have recently become a new environmental problem [5]. Many studies have been performed assessing the impact of hospitals and their contribution to the pharmaceutical loads found in wastewater [6-8], the efficiency of the removal of pharmaceuticals by wastewater treatment plants (WWTP) [9], the spatial and temporal differences of pharmaceuticals occurrence in wastewater [10] and recently to compare the results of the wastewater analysis with predicted concentrations based on prescription figures [11, 12]. In the context of sewage-based epidemiology (SBE) pharmaceutical residues also offer the potential to map certain types of disease and other health based stressors[13].

The measurement of illicit drugs and pharmaceuticals in wastewater has become an extended topic over the past decade and SBE has been established as a complementary approach for monitoring trends of illegal drug use [14-18]. Recently the approach has been evaluated with an international study comparing the drug consumption in 21 different countries by quantitatively analyzing illicit drugs and their metabolites in 47 WWTP [19, 20]. SBE has been used to estimate drug use in communities (i.e. city, small town, school, prisons) as well as identify changes in drug consumption during special events [21, 22], temporal changes [17], and providing complementary data on the use of NPS [23-25]. Thus far, the analytical methods used for SBE have focused on the principal challenges related to the low concentrations of drug residues in wastewater combined with the complexity of the wastewater matrix. These procedures have been mainly developed for classic illicit drugs (cocaine, amphetamines, opioids and cannabis) and a broad range of pharmaceuticals, and based on solid-phase extraction (SPE) for sample pre-treatment and pre-concentration followed by instrumental analysis, typically liquid chromatography coupled to tandem mass spectrometry (QqQ)[5, 16].

The hyphenation of liquid chromatography to high resolution mass spectrometry (HRMS), i.e. orbitrap and quadrupole time-of-flight (QTOF), has increased the potential of SBE to identify NPS and their residues in sewage based samples. HRMS offers some unique advantages for screening and identification, such as better specificity due to its increased resolving power and the possibility to perform retrospective analyses without the need of additional sample analyses [26].

Since the NPS market is in constant flux, new strategies are urgently required. New analytical methods based on QqQ for the target analysis of NPS are continuously being published[24, 25] but in general these techniques cannot keep up with the rapidly evolving NPS market making necessary the development of new approaches that combine target analysis with wide-scope suspect screening capable of confirming the presence/absence of a new compounds with quantification should reference standards be available[27]. This challenge can be overcome by using a suspect screening approach that has already been demonstrated as a suitable technique for the detection and confirmation of several organic compounds[27], including NPS[23, 28]. The simultaneous determination of a broad number of compounds in one injection, with a corresponding reduction of time and costs, without the need for reference standards make this approach one of the current trends in environmental analytical chemistry.

The aim of the presented work was to establish a broad analytical tool for the analysis of different types of drugs in sewage-based samples, such as pooled urine, wastewater and passive sampler extracts and develop a dynamic workflow for the introduction of new substances of interest in the future due to the high potential of the HR-MS instruments for the identification of suspect compounds presented in the sample.. Firstly a targeted multi-residue method for the simultaneous identification and quantification of 51 drugs, pharmaceuticals and their main urinary metabolites by UHPLC/QTOF was validated as recommended by EU guidelines with minor modifications [29, 30]. With the target method established to track the quantitative occurrence of selected target analytes, the secondary goal of this work was to detect possible NPS and other compound of possible interest in sewage-based samples thought the use of a suspect screening approach. The present work also describes the potential of non-target analysis for identifying NPS and their subsequent inclusion into the suspect database or target method if standard reference is available through a dynamic approach.

2. Materials and methods

Detailed information relating to reagents and chemicals (illicit drugs, pharmaceuticals and metabolite reference substances), the sewage-based samples and extraction procedure can be found in the Supplementary Information (**SI**).

2.1 Sewage-base samples

Influent samples were collected from two Norwegian WWTPs; VEAS WWTP in Oslo and Ladehammeren and Høvringen WWTP in Trondheim. The robustness of the method was also examined by analysing different sewage-based samples. The "Pharmaceuticals" version of the polar organic chemical integrative sampler (POCIS) was placed for two weeks in the sedimentation overflow channel at the Oslo WWTP and the pooled urine samples were collected from three different Norwegian music festivals. **Figure 1** shows an overview of the samples used in this study (detailed information provided in **SI**).

2.2 Extraction procedure

Defrosted sewage samples (100 mL) were spiked with a mix of 25 isotope labeled internal standards (ILIS) to give a concentration of 400 ng L^{-1}. The pH was controlled but generally was the desired (~7). Subsequently, extraction was performed using a SPE-DEX fully automatable extraction system (Horizon Technology, Salem, NH, USA) using HLB (Hydrophilic-Lipophilic Balance) extraction disks (47 mm, I.D.; Horizon Technology, City, Country) (more information in **SI** and **Table S-1**).

The selection of the Oasis HLB extraction sorbent was based on previous experience. Oasis HLB and MCX generally yield the highest recoveries for most of the drugs and pharmaceuticals [5, 18]. Compared with Oasis MCX, HLB provides a lower selectivity for some basic compounds, such as amphetamine-like compounds and a considerable increase of the matrix components retained in the sorbent leading to a higher sensitivity. Despite this, HLB, with a mixed-mode cation exchange sorbent, offers the possibility to extract a wide range of compounds with different psychochemical characteristics suiting the goal of the method, enabling the simultaneous analysis of the widest range of drugs and pharmaceuticals in one single extraction.

To determine the method recovery influent wastewater from VEAS WWTP in Oslo was spiked in quintuplicate with a standard mixture containing all of the target analytes at two different concentrations together with a mixture of the 25 ILIS. The two concentrations were selected according to those generally found in wastewater influent.

2.3 Ultra-high pressure liquid chromatography

The chromatography column, stationary phase and mobile phases were selected in accordance with a pre-established screening method[31]. **Figure 2** shows the 2D mass chromatogram of the low energy channel (6eV ESI^+) for the optimal separation of all target compounds and NPS at a concentration of 250 ng mL^{-1}. A Waters Acquity UPLC system (Milford, MA, USA) was used for this work. Chromatographic separation was carried out using an Acquity UPLC HSS C18 column (2.1 x 150 mm, particle size 1.8 µm) (Waters, Milford, MA, USA). Gradient elution was performed at a constant flow of 0.4 ml min^{-1} using 5 mM ammonium formate, pH

3.0 (solvent A) and acetonitrile with 0.1 % formic acid (solvent B). The gradient elution starts with 87% A and then increasing B to 95% in 15 minutes: Solvent A, held for 0.5 min; 0.5-10 linear rate to 50% B, 10-10.75 linear rate to 95% B, held for 0.5 min; reconditioning with a linear rate to 87% A, 12.50-15 min. The analytical column and the guard column were kept at 50 ºC and the sampler manager at 5 ºC. The weak and strong wash used to remove the contaminants from the needle and the injection port were 10% ACN in water (600 µL) and 95% ACN in water (200 µL) respectively.

2.4 Quadrupole Time-of-flight Mass Spectrometry

A Xevo G2-S Q-TOF mass spectrometer (Waters, Milford, MA USA) was used in positive ESI mode for acquisition using MS^e, that allows both precursor and product ion data to be simultaneously acquired during a single run. The MS method consists of 3 functions, the first (low energy, LE) applies collision energy of 6 eV, the second function (high energy, HE) acquires through a collision energy ramp of 15-50 eV and the third function acquires the lock mass data for online mass calibration. The MS range is 70-700 with a scan time of 0.1 second in continuum mode, preserving the peak shape of the exact-mass precursor and product ions. The source conditions whose maximum intensities were achieved were the following: capillary voltage 3 kV, sample cone, 20 V, source offset 80 V, source temperature 120 ºC, desolvation temperature 500 ºC, cone gas flow rate 50 L h^{-1}, desolvation gas (N_2) flow rate 1000 L h^{-1}.

The mass spectrometer was calibrated using a solution of sodium formate over a mass range of 50-1000 Da. Analyses were performed using an external reference (Lock-Spray™). During the data acquisition the mass was corrected using an external reference (Lock-Spray™) consisting of 0.2 µg mL^{-1} solution of leucine-enkephalin infused continuously at 10 µL min^{-1} via a lockspray interface. The lock mass data were acquired every 20 seconds for 0.1 seconds and for the rest of the time the baffle in the ion source blocked the entry of the lock spry. This generated a reference ion in positive mode at *m/z* 556.2771 that was used for real-time mass corrections in order to maintain the mass accuracy and reproducibility.

2.5 Data processing

The workflow and identification confidence used in this work were based upon those described by Krauss et al. [32] and Schymanski et al. [33](**Figure 3**).

The MSe data processing using the UNIFI screening platform (Waters Corporation, Milford MA, USA) was performed in two steps. Firstly, all the continuum data was peak detected using a 3D peak algorithm based on the calculation of the peak volumes by the detection of all the ion crests in a given mountain range. This provides a complete list of retention-time / *m/z*-pairs which are then used for screening. The second step which follows peak detection is the screening protocol where Unifi matches the observed *m/z* retention-time pairs

against the scientific database using defined settings such as the retention time accuracy (2.5 %), mass accuracy of parent ion and fragment ions (5 ppm) and theoretical isotope patterns for the protonated molecule at LE and at least two accurate fragment ions at HE [30]. The database is built from a pre-established screening method [31] which uses the same chromatographic and detector parameters as in this study.

For target screening the procedure is unchanged, except the screening is focused only on the 51 target compounds (as opposed to the entire 1000 + library) and reference standards are run with the analysis to prove quantitative information/results (**Table 1**). Protonated precursor ion data at LE was extracted and used for quantification. For this work, selection of the 51 target analytes was based on their reported use in Norway and their previously reported occurrence in wastewater [17, 19, 24].

For suspect screening, the retention-time/ m/z-pairs are compared against a broad list of approximately 1000 compounds (note that this database is under constant renewal and expansion so the exact number of compounds varies over time). UNIFI also automatically assesses the candidate values with the fragments, mass defect, isotope pattern and adducts (M+H, M+Na, $M+NH_4$). The suspect screening database (including mass spectra, assigned fragments and retention time) was applied under the same acquisition parameters as above and tested in this work on pooled urine samples in an attempt to identify NPS or possible compounds of interest to include in the future in the target method through a reiterative development workflow.

While the current study used a screening database developed around a commercially product, and manually augmented with 16 NPS, suspect screening may also be performed using other public databases (e.g. MassBank, Chemspider) or in-silico fragmentation tools (MetFrag) which are available for many environmental contaminants. Suspect screening lists may also be produced with computational prediction systems that can provide a list of potential excreted biomarkers and biotransformation products in wastewater [34].

Directly infusing the standards and subsequently analyzing the spectra usually calculate fragmentation pathways. This study attempts to complement the suspect-screening database by importing suspect candidates relying on accurate spectral information and different non-target tools. Where analytical data on fragmentation is lacking the UNIFI fragment match tool applies a series of novel algorithms based on systematic bond disconnections of the precursor structure to predict fragmentation pathways that can then be compared with measured spectra in order to help to increase the amount and the reliability of the information essential for the subsequent identification and confirmation of the compound of interest. This approach has been tested in this study with two NPS. The common fragment approach is another interesting option for the detection of NPS. Synthetic cannabinoids generally appear on the market with minor structural modifications which means that

during fragmentation they lose the same specific functional groups and that structural information can be used as a filter to detect those components. This tool has been tested with 14 different synthetic cannabinoids.

2.6 Quantification and method validation

The performance of the method was evaluated following EU guidelines with minor modifications[29, 30]. The instrumental linearity of the method was studied by analyzing standard solution in triplicate at 9 different concentrations that were between 0.25 ng mL^{-1} and 500 ng mL^{-1} (it would be equivalent to 1-2000 ng L^{-1} in sewage after applying the pre-concentration factor). Satisfactory linearity was assumed when the correlation factor (R^2) was > 0.99, based on analyte/internal standard peak area ratio applied in different concentration ranges for each compound. The most relevant aspects about the instrumental parameters are summarized in **Table S-2**.

The theoretical limit of quantification (LOQ_{THE}) for real samples was calculated based on the ratio between the instrumental limit of quantification, estimated as a signal-to-noise (S/N) ratio of 10 for the standard in the calibration curve at the lowest level, and the matrix suppression (MS) for each deuterated internal standard multiplied by the concentration factor (CF).

$$Theoretical\ LOQ_{RS} = \frac{LOQ_{inst}}{MS} x\ CF$$

The matrix effect defined as Matuszewski et al.[35] was calculated as the ratio between the accurate mass signal of the 25 deuterated standard spiked in different sewage samples after extraction and the accurate mass signal of the same deuterated standard in the calibration curve. The influence of the sewage matrix concentration on the analysis is a crucial factor but simply controlled with a pre-concentration factor study[36]. For analytes where deuterated standards were unavailable, either the deuterated analyte with similar structure, polarity or the closest retention time was selected for correction.

Precision was expressed as the repeatability of the method in terms of relative standard deviation and trueness was tested by means of the absolute recovery due to the lack of quality control standards. The relative recoveries and standard deviations of the whole procedure were calculated by analyzing sewage water samples spiked at a low and high concentration with the analytes (100 and 600 ng L^{-1}) according to the levels generally found in wastewater and with 600 ng L^{-1} of the deuterated internal standard solution mix. All recovery experiments were performed in six replicates (n=6). Concentrations of target compounds found in wastewater "blank" samples were subtracted from the spiked samples due to the absence of an appropriate wastewater blank. Instrumental accuracy and precision was assessed by analyzing spiked Mili-Q water (n=5) at two

different concentrations for each compound, low level (between points 2-3 on the calibration curve) and high level (between points 8-9 on the calibration curve).

The stability of the target analytes in wastewater was evaluated in triplicate (n=3) via the analysis of 500 mL of spiked (1000 ng mL^{-1}) wastewater immediately following spiking (t=0 hours) and again at t=5, 8 and 120 hours post spiking. The wastewater sample (pH 7.5) was stored in an amber glass bottle at 10 ºC throughout the study in order to best replicate the average wastewater influent temperature.

3. RESULTS AND DISCUSSION

3.1 Sample extraction optimization

Selected parameters were investigated in order to optimize the method. Silanisation of the glassware, by deactivating the ion-exchange groups with dichlorodimethylsilane, resulted in increased recoveries for some of the more polar drugs, such as amphetamine-like compounds (Figure S-1). Elevated evaporation temperature was also found to result in analyte losses, therefore special attention was paid to the drying of extracts .Figure S-2 shows poor recoveries for some of the phenethylamines following nitrogen evaporation at 50°C. All samples were subsequently evaporated to around 100 µL under a gentle nitrogen stream at 35 ºC.

3.2 Method performance

The relative recoveries and standard deviations from the relative responses analyte/ILIS are shown in the **Table S-3**. For the target analytes with corresponding ILIS the recoveries are typically >80% with some exceptions such as MDMA, norketamine and atomoxetine with slightly lower recoveries of around 70%. The remaining target analytes were corrected using the ILIS with the closest structure, polarity or eluting time, achieving relative recoveries ranging from 60 to 118%. However certain analytes such as clonazepam (56%), dehydronorketamine (53%), methoxetamine (40%) and methylphenidate (50%) showed lower recoveries. Relative recoveries at 100 ng L^{-1} are in general slightly lower due their proximity to the LOQ_{THE}, however in general the recoveries do not substantially differ between the low and high concentration. The overall method precision was calculated as the relative standard deviation (RSD) due to the lack of reference materials for the wastewater samples, and showed satisfactory results <20% RSD for all compounds.

Paracetamol and hydroxycotinine were detected at high concentrations in the blank (low ppb) making subtraction and the recovery calculation not possible for paracetamol, and less precise for hydroxycotinine (highest RSD). Carbamazepine also exhibits ionization issues in terms of signal enhancement with recoveries slightly higher than 100% while other compounds with high signal suppression were gabapentin, *p*-

hydroxymethamphetamine, MDA, diclofenac, THC-OH and THC-COOH, which were finally excluded from further validation in this study, but this does not however preclude the potential for lower matrix effects and better analytical outcomes in wastewater from other locations or other sample types that present a cleaner matrix.

The overall method recoveries were satisfactory and similar to those screening methods previously reported for pharmaceuticals in wastewater samples [5]. Different target procedures, such as QqQ, with optimized and specific conditions for each analyte, generally present greater sensitivity. This is a downside of all multi-residue methods where the optimized conditions are compromised in order to accommodate several analytes, especially in complex and variable matrix such as wastewater. Nevertheless the good sensitivity levels and dynamic range presented in the new HR-MS instruments together with the instrumental performance in terms of repeatability and sensitivity helps to counteract low recoveries.

3.3 Quantification and method validation

Table S-2 provides an overview of the performance of the developed target method. The instrumental linearity for nine concentration levels is in the range of 0.25 to 400 ng mL^{-1}, equivalent to 1-2,000 ng L^{-1} in wastewater after applying the pre-concentration factor. These are within the levels reported in literature[16] showing good results for all of the initial 51 selected compounds presenting correlation coefficients greater than 0.99. Different ranges were applied for every compound according to sensitivity. Instrumental LOQ was calculated as the signal-to-noise (*S/N*) ratio of 10 for the standard in the calibration curve at the lowest concentration. Instrumental accuracy and precision, both for intra- and inter-day, was assessed at two different concentration levels for each compound showing an satisfactory accuracy levels between 87.8-113.1 % range and precision <12.8 % (RSD).

The theoretical limits of quantification (LOQ_{THE}, **Table S-3**), calculated based on the ratio between the instrumental limit of quantification and matrix suppression multiplied by the concentration factor for each deuterated internal standard, varied between 0.4 and 187 ng L^{-1}. This is just a valuable estimation about the method performance and due to the complexity of the matrix common sense has to be used prior the analysis of a new batch of samples.

The instrumental performance was also evaluated in terms of the intra-day (repeatability) and inter-day (reproducibility) precision studies from five spiked Mili-Q water samples at two different concentration levels in five repeated injections and three consecutive days. The results in terms of RSD show values below 10% in both intra and inter-day studies for all the compounds except for THC-COOH at the low concentration level for the intra-day (10.2%) and amphetamine and flunitrazepam at the low concentration level for the inter-day (11.8

and 12.8 respectively). The good instrumental performance together with the high selectivity reached and low mass error for all the target compounds (mass error range between -0.51 and 0.39 mDa, (**Table S-2**) ensure the reliability of the instrument performance, an essential aspect in HRMS.

3.4 Liquid Chromatography-Mass Spectrometry

Suppression or enhancement of the target ion signal in LC-MS is a critical issue in wastewater analysis due to the complex nature of the sample and the co-elution of the analytes with matrix constituents. The negative consequences of this problem are best avoided with the use of appropriate ILIS, together with extensive sample pre-treatment (clean-up), chromatographic separation and sample dilution. The effects of the sample matrix was evaluated by comparing the matrix effect/pre-concentration factor ratio for all the 25 deuterated internal standards. Deuterated internal standards are not present in sewage water samples but are affected by the same potential losses during the analytical process as the analyte of interest. The influence of the SPE loading volume on matrix suppression was optimized by spiking 100 ng of the deuterated internal standard mix solution into the extracts of 3 different wastewater extracts at 4 different volumes (100, 200, 300, and 400 mL). The accurate mass signal was compared with the accurate mass signal of the deuterated internal standards in solvent. A loading volume of 100 mL was found to give the most satisfactory compromise for the analysis with a pre-concentration factor of 250 (LC vial volume 400 µL, injection volume 5 µL) (**Figure S-3**).

Van Nuijs et al.[37]considered not including matrix effect studies in method validation, claiming that matrix effects can be highly variable between samples. We agree on the fact that each individual sample has a different composition, but we strongly recommend the matrix characterization of different batches of wastewater samples when they come from different locations or affected by different conditions since an appropriate sample dilution can improve substantially the general performance of the method. Generally analytical methods rely on the validated LOQ but wastewater analysis required a more intense day-to-day control.

3.5 Stability in wastewater

The stability of the analytes in wastewater was investigated to ensure the use of appropriate sample-storage and handling procedures. The study was configured to estimate the degradation of our target compounds during the in-pipe transit time for a single wastewater sample.

Table S-4 shows the results for the stability test for the target analyte signal in non-filtered wastewater at pH 7.5 and 10°C expressed as the difference between initial and final concentrations at different time points (t= 0, 5, 8 and 120 hours). Time points were set according the mean residence time in the sewer system from Oslo to

304 the VEAS WWTP, 5 hours, the total treatment time in the plant, 3 hours, and finally a long exposure during
305 120 hours to provide an estimation of the biodegradation after 5 days.

306 Over the first 5 hours EDDP is the only analyte with major change in peak area (45% higher). Between 5 and 8
307 hours significant changes were also observed for hydroxycotinine, benzoylecgonine, methylphenidate the
308 concentration changed substantially (>±30 %). The results following 120 hours show degradation for the many
309 of the target analytes, such as morphine (83%), *p*-hydroxymethamphetamine (70%), paracetamol (-88%), MDA
310 (-96%), methylphenidate (-88%), cocaine (-75%), EDDP (77%), nitrazepam (-79%) and phenazepam (-70%).

311 These data provide an indication of the transformation that may occur during in-sewer transport. The results are
312 in accordance with previous reports [38].

313 3.6 Application to sewage water samples

314 3.6.1 Target Screening.

315 The main objective of this work was to demonstrate the broad applicability of a multi-residue method in MS^e
316 by UHPLC-QTOF for both screening and quantification purposes. The validated method was applied to the
317 analysis of 15 wastewater samples collected from two WWTPs in Oslo and Trondheim in 2014 to assess the
318 applicability of the method. **Table 2** shows the population normalized loads for the target drugs in wastewater,
319 estimated from the daily concentration of each detected target compound in the wastewater sample, daily flow
320 discharged into the WWTP and by the population size (mg day^{-1} 1000 $inhabitants^{-1}$). Results from Oslo show
321 the daily average loads calculated from the analysis in triplicate of a daily composite sample taken every
322 Friday, Saturday and Sunday during three consecutive weekends.

323 In general, pharmaceuticals were detected in higher concentration for both cities. Atenolol, paracetamol,
324 metoprolol, propranolol, citalopram, carbamazepine, oxazepam were detected at elevated concentrations in all
325 of the samples from Oslo and Trondheim. Methylphenidate and alprazolam were only present in samples from
326 Trondheim, suggesting different levels of consumption within the same country.

327 The heroin metabolites, 6-MAM and morphine, were detected in samples from both locations but it is
328 important to remember that while 6-MAM is specific to heroin, the presence of morphine in wastewater also
329 can result from the clinical use of legal pharmaceuticals containing morphine or codeine. Methadone and its
330 metabolite, EDDP, are usually present in wastewater samples and in this study the loads obtained in Oslo are
331 approximately ten times higher than those from Trondheim. Biomarkers of other commonly used drugs, such as
332 cocaine, benzoylecgonine, amphetamine and methamphetamine were detected in samples from both cities at
333 different concentrations. While the loads of cocaine/benzoylecgonine are approximately ten timers higher in
334 Oslo than in Trondheim, the results for amphetamine and methamphetamine are slightly higher in Trondheim

suggesting different use patterns. This is in agreement with the general geographical distribution of the drugs in Norway published in the Annual report to the European Monitoring Centre for Drugs and Drug Addition[39] where cocaine seems to be a big city phenomenon with special relevance in Oslo and at the same time within the European context Norway has been shown to have one of the biggest market for amphetamine/methamphetamine[39].

Cocaethylene, the main urinary biomarker of cocaine co-consumption with alcohol, and MDMA were identified and quantified only in Oslo. Methylone, ketamine and methoxetamine were found in at least one of the composite samples from Oslo suggesting a sporadic weekend use.

This method is also been successfully used for the analysis of other sewage-based samples, such as pooled urine samples and passive sampler extracts. Sample preparation was adapted for each case (**SI**). It is worth mentioning the importance of the dilution factor for both POCIS and pooled urine samples since the concentration level for the target biomarkers are extraordinary high compared with those typically found in wastewater, being a very suitable samples for suspect screenings.

Table S-5 and S-6 show the results of the target analysis performed on 10 POCIS extracts from the winter of 2013 and three pooled urine samples collected from three different music festivals in summer of 2014.The results for the passive sampling devices must be corrected with an exposure factor, however this is outside of the scope of this paper and therefore the results are presented as amount of analyte (ng) per POCIS. The target method was also used for the analysis of the pooled urine samples collected from music festivals.

In general the compounds identified in the POCIS do not differ with the compounds obtained and reported in this study for the wastewater samples, however in the pooled urine samples the differences are greater, especially for the amphetamine-like compounds exposed to a lower dilution factor compared with the other sewage-based samples. The low dilution factor makes pooled urine samples a very suitable sample for suspect screening purposes.

3.6.2 Suspect Screening

The suspect screening database was applied to the pooled urine samples to demonstrate the applicability of these libraries. The aim of this work was to develop a reiterative tool to track the presence of the target compounds in wastewater by complementing the 51 target analytes with new targets identified via suspect screening The 51 compounds were selected as described above, however as shown in **Table 2** not all were present or detected in the sewage-based samples. Suspect screening together with retrospective analysis allows the selection of new compounds, based on detection in the wastewater to be added to the quantitative target

method through the use of an authentic reference standard thereby facilitating decision-making and improving cost effectiveness.

The continuum data for the three-music festival samples were peak detected using the processing settings described above. Following peak processing UNIFI provided a list of *m/z* values termed as candidates. The number of candidates for the samples were 11,220, 11,183 and 11,164 for each festival. The acceptance criterion for the tentative candidates is within the tolerance range described in the data processing section. Despite this, in this method the authors defined limit checks broader to perform the final step manually, meaning that after the evaluation of the candidates against the database UNIFI provided a new list with 182, 151 and 131 tentative IDs respectively. Table S-7 shows some examples of positive identifications for the ID's fulfilling the criteria consisted of a mass accuracy ±5 ppm, retention time ±0.5 min, isotope match ±5 ppm and a minimum of two detected ions in the HE. Sildenafil, a medicine to treat erectile dysfunction, zolpidem, prescription medication for the treatment of insomnia, and benzocaine and lidocaine, known cocaine cutting agents are interesting for future target analysis. In this case the 24 compounds identified (Table S-7) would move from the level 3 (tentative candidates) to the level 2 (probable structure) in the absence of the reference standards for the final confirmation [33].

In order to augment the suspect database used in this study, a non-target approach was used to net purchased NPS; 5/6-APB and N-adamantyl-1-pentylindole-3-carboxamide (commercial names: Benzo Fury and 2NE1-APICA). **Figures S-4 and S-5** illustrate the detection and identification of the 5/6-APB and N-adamantyl-1-pentylindole-3-carboxamide. UNIFI automatically detects the sample components, and subsequently through the accurate mass spectra allows the identification of the unknown candidates. In contrast with the characterization analysis of the classic drugs, the NPS chromatograms are relatively clean since they are purchased in almost pure form without any additive or "cutting agent" as is shown in **Figure S-4** and **S-5** where only two peaks are present as the most abundant. As an example, **Figure S-5** shows a chromatogram with the assigned fragments (159.0798 and 131.0488) from the accurate mass ion 176.1065 at 5.2 min.

A common fragment approach was used also used as a complementary tool for the identification and subsequently introduction of 14 synthetic cannabinoids into the suspect database. **Figure S-6** shows the total ion chromatogram of the 14 synthetic cannabinoids described in the picture together with the chromatogram of the common fragment m/z 155.0492 belonging to 8 of the synthetic cannabinoids.

4. CONCLUSIONS

A high confidence multi-residue method using auto-SPE-UHPLC-QTOF for the determination of 51 drugs, pharmaceuticals and metabolites was validated and applied to different sewage-base samples. Meanwhile, this analytical procedure is also intended to fulfill the analytical challenge of the detection and reliable identification of NPS appearing in the market through the application of a suspect screening approach augmented by a reiterative process intended to both update the target multi-residue method, and the suspect screening list.

The selection of HLB as an extraction sorbent and C18 as the chromatography column provides a broad and generic methodology for the rapid identification of a larger number of compounds dealing with the compromise between elimination of interfering matrix components (using more selective extraction procedures) or the unification of all requested features in just one method. The method has a satisfactory linear dynamic range and the sensitivity. Even though many papers have shown the capability of the HRMS screening techniques just for qualitative purposes, the high selectivity, accurate mass and the information provided by the fragments as mass defect or the isotopic pattern, make this method in a very reliable tool also for the quantification of drugs and emergent substances in wastewater.

The described reiterative and dynamic workflow has been designed in order to constantly update the screening levels based on experimental data (**Figure 3** Target analysis can be supplemented by simultaneously screening through the suspect database in order to identify new compound of interest, all in one injection. If a suspect candidate is identified, this candidate can be later confirmed by purchasing the reference standard and this compound can then be incorporated into the target method. Despite not being used in this work, non-target screening is an additional avenue of the described workflow which can enhance the potential of the approach. The application of this method was finally tested with different real samples showing an extensive list of confirmed compounds in different sewage-based samples confirming the broad applicability of the developed method. Two NPS were also introduced into the suspect database together with another 14 synthetic cannabinoids as part of the strategy to expand the library for further identification purposes in the future.

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Figures and Tables

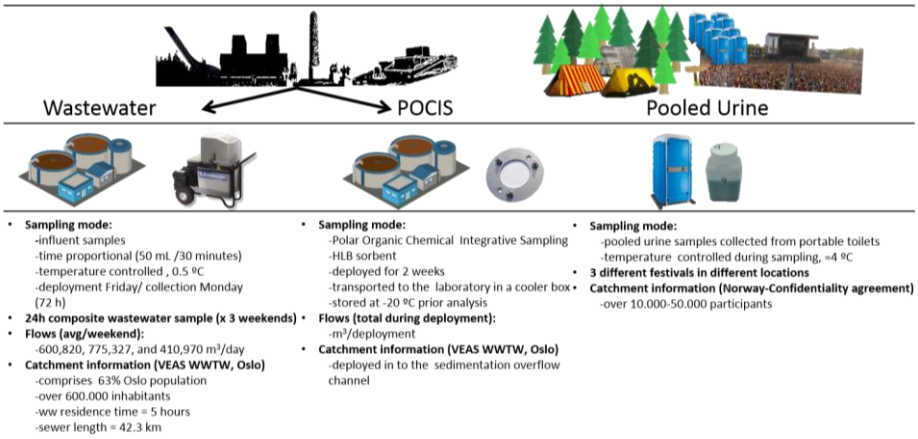

Figure1

Different sewage-based samples used in this study

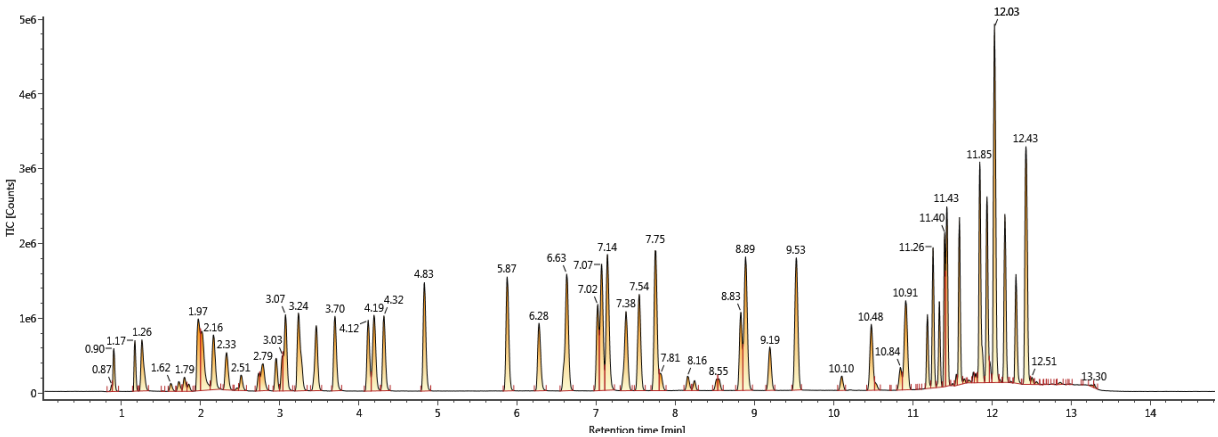

Figure 2

2D mass chromatogram of the low energy channel (6eV ESI+) of a 100 ng/mL standard mix solution with all the target compounds, UNIFI (Waters Corporation, Milford MA, USA)

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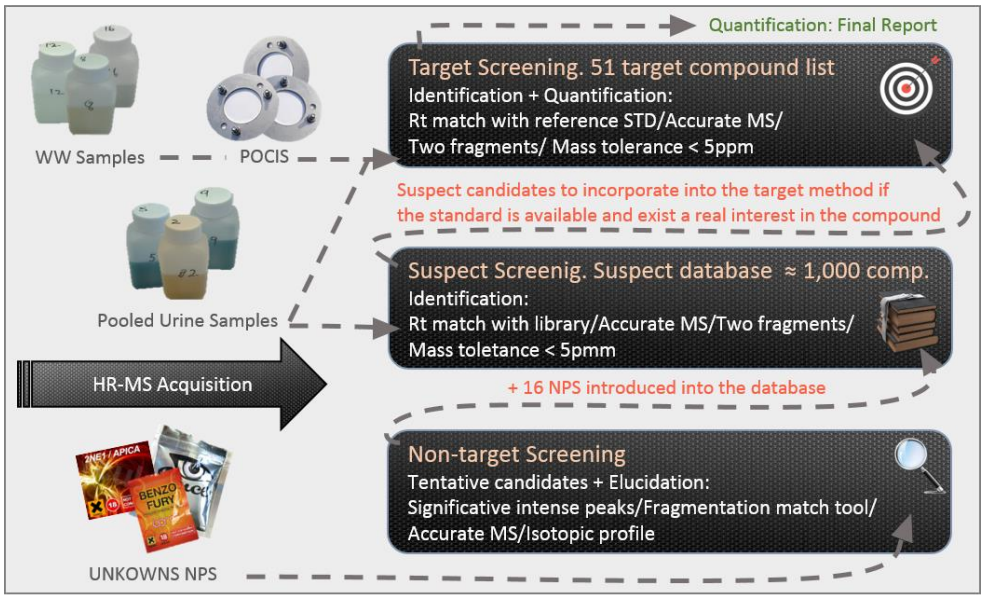

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585 **Figure 3. Illustrative workflow diagram for the three different approaches developed in this study.**

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589 **Table 1**

590 **Target compounds organized by their retention times with the experimental parameters.**

Compound	*Internal Standard*	t_R	*Precursor ion*	*Product ion*	*Linear range*
			[M + H]+	*(m/z)*	*(ng mL^{-1})*
Morphine	Atenolol-d_7	1.17	286.14376	201.0910	0,5-100
Hydroxycotinine	Atenolol-d_7	1.17	193.09714	134.0595	0,5-400
Atenolol	Atenolol-d_7	1.26	267.1703	190.0863	0,5-200
Salbutamol	Atenolol-d_7	1.28	240.1594	145.0648	1-100
p-Hydroxymethamphetamine	Atenolol-d_7	1.31	166.12263	135.0798	2-100
Paracetamol	Atenolol-d_7	1.62	152.07059	110.0601	2-400
Cathinone	Methcathinone-d_3	1.75	150.09133	132.0808	5-400
Gabapentin	Gabapentin-d_{10}	1.8	172.13319	154.1227	5-200
Pseudoephedrine	Methcathinone-d_3	1.96	166.12263	133.0886	5-400
Methcathinone (Ephedrone)	Methcathinone-d_3	1.98	164.10698	146.0971	1-400
Methylone	Methylone-d_3	2.16	208.09681	160.0757	0,25-400
6-MAM	Methylone-d_3	2.29	328.15432	165.0699	0,5-400
Amphetamine	Amphetamine-d_5	2.41	136.11207	91.0543	5-400
MDA	MDA-d_5	2.47	180.10189	163.0754	10-200
PMA	MDMA-d_5	2.53	166.12263	91.0543	2-400
Methamphetamine	Methamphetamine-d_5	2.72	150.12771	119.0856	2-200
MDMA	MDMA-d_5	2.75	194.11754	163.0754	2-400
Dehydronorketamine	Norketamine-d_4	2.89	222.06801	205.0415	1-400
PMMA	MDMA-d_5	2.98	180.13828	121.0648	2-400
Benzoylecgonine	Benzoylecgonine-d_3	3.07	290.13867	168.1020	0,25-400
Mephedrone (4-MMC)	Mephedrone-d_3	3.19	178.12263	160.1127	2-400
Norketamine	Norketamine-d_4	3.21	224.08366	125.0153	5-200
Ketamine	Ketamine-d_4	3.45	238.09931	125.0153	0,5-400
4-MEC	Ketamine-d_4	3.7	192.13828	174.1278	0,5-400
Methoxetamine	Metoprolol-d_7	4.12	248.16449	121.0648	0,25-400
Metoprolol	Metoprolol-d_7	4.19	268.1907	116.1070	0,25-400
Methylphenidate	Metoprolol-d_7	4.32	234.14884	84.0808	0,25-400
Cocaine	Cocaine-d_3	4.83	304.15432	182.1176	0,25-200
Cocaethylene	Cocaine-d_3	5.87	318.16997	196.1333	0,25-400
Propranolol	Fentanyl-d_5	6.28	260.16449	116.1070	0,25-200
Fentanyl	Fentanyl-d_5	6.63	337.22742	188.1434	0,25-400
AH-7921	Fentanyl-d_5	7.02	329.11818	284.0611	0,25-400
Citalopram	Buprenorphine-d_4	7.07	325.17105	109.0449	0,25-200
Midazolam	Buprenorphine-d_4	7.14	326.08547	291.1167	0,25-400
Buprenorphine	Buprenorphine-d_4	7.38	468.31081	414.2633	0,25-400
Carbamazepine	Buprenorphine-d_4	7.54	237.10223	194.0965	0,25-200
EDDP	Atomoxetine-d_7	7.75	278.19031	234.1277	0,25-400
Atomoxetine	Atomoxetine-d_7	7.81	256.16958	148.1103	1-200
Nitrazepam	Oxazepam-d_5	8.16	282.08731	236.0945	1-400
Oxazepam	Oxazepam-d_5	8.22	287.05817	241.0528	2-400
Clonazepam	Oxazepam-d_5	8.5	316.04833	270.0555	1-400
Lorazepam	Oxazepam-d_5	8.6	321.0192	275.0138	5-400
Alprazolam	Oxazepam-d_5	8.83	309.09014	281.0715	0,25-200
Methadone	Methadone-d_3	8.89	310.21652	265.1587	0,25-400
Flunitrazepam	Diazepam-d_5	9.19	314.09353	268.1007	1-400
Etizolam	Diazepam-d_5	9.53	343.07786	314.0391	0,25-200
Phenazepam	Diazepam-d_5	10.1	348.97382	196.1706	2-400
Diazepam	Diazepam-d_5	10.91	285.07891	193.0886	0,25-200
Diclofenac	Diazepam-d_5	11.4	296.02395	215.0496	5-400
THC-OH	THC-OH-d_3	11.83	331.22675	313.2162	5-400
THC-COOH	THC-COOH-d_3	11.85	345.20602	299.2009	5-400

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Table 2

Loads (mg day-1 1000 inhabitants-1) of detected analytes in influent wastewater samples from the Oslo (mean ± SD) and Trondheim (mean, n=1) WWTPs

a founded it at least one of the daily samples (not presented in all)

	Oslo, February 2014						Trondheim, Spring 2014					
Analyte	*1st weekend*		*2nd weekend*		*3rd weekend*		*Friday 28.02.14*	*Saturday 08.03.14*	*Friday 21.03.14*	*Saturday 29.03.14*	*Friday 04.04.14*	*Saturday 12.04.14*
Morphine	48.2	±12.3	41.8	±13.4	47.5	±9.2	6.1	7.2	8.7	5.5	13.3	12.3
Hydroxycotinine	3873.8	±616.9	1919.1	±204.9	2155.9	±243.6	239.5	331.7	500.1	577.7	575.3	636.3
Atenolol	165.7	±22.0	134.3	±21.1	136.2	±6.5	45.0	75.3	53.3	53.2	95.8	77.1
Paracetamol	6323.7	±1026.7	6335.2	±153.2	4210.4	±468.2	485.6	1129.3	1703.1	1481.7	2355.3	2209.2
Methylone[a]	11.8	-	nd	-	nd	-	nd	nd	nd	nd	nd	nd
6-MAM	25.2	±10.5	6.0	±10.4	2.8	±2.4	8.0	16.1	25.3	23.7	32.6	39.2
Amphetamine	51.5	±10.8	38.1	±7.6	51.1	±4.1	76.5	124.1	91.2	53.2	91.8	56.9
MDMA	75.5	±65.5	25.7	±22.3	26.8	±23.8	nd	nd	nd	nd	nd	nd
Methamphetamine	<LOQ	-	26.3	±9.7	25.8	±2.4	14.2	26.3	11.6	35.2	50.1	49.6
Benzoylecgonine	214.3	±86.8	216.3	±24.1	240.9	±59.1	3.5	14.5	5.8	9.0	9.0	12.2
Ketamine[a]	nd	-	5.3	±9.1	nd	-	nd	nd	nd	nd	nd	nd
Methoxetamine[a]	0.8	±1.3	nd	-	nd	-	nd	nd	nd	nd	nd	nd
Metoprolol	836.2	±87.5	859.1	±183.9	857.3	±64.0	123.0	125.6	141.8	146.3	280.1	250.2
Methylphenidate	nd	-	nd	-	nd	-	0.6	1.2	2.7	1.3	2.7	3.0
Cocaine	92.5	±35.5	77.1	±18.5	88.7	±23.6	0.8	2.1	3.1	2.0	3.4	4.0
Cocaethylene	7.3	±6.7	9.8	±1.3	6.1	±1.7	nd	nd	nd	nd	nd	nd
Propranolol	3.5	±0.7	14.8	±1.5	0.8	±0.1	4.3	13.9	20.8	13.0	22.7	26.7
Citalopram	44.0	±9.1	49.3	±7.7	44.8	±0.9	32.2	43.9	14.5	12.3	27.5	17.4
Buprenorphine	nd	-	nd	-	nd	-	214.5	292.5	96.7	81.8	183.5	115.7
Carbamazepine	258.3	±29.2	294.6	±16.6	248.0	±27.1	52.1	52.2	35.0	69.2	116.7	112.7
EDDP	48.5	±9.2	36.3	±3.5	18.6	±1.9	10.3	11.6	7.2	7.1	13.9	8.6
Oxazepam	153.2	±32.3	125.0	±25.9	144.0	±6.5	72.6	119.8	69.3	94.7	141.4	140.9
Alprazolam	nd	-	nd	-	nd	-	7.5	798.8	462.0	631.0	942.5	939.1
Methadone	13.4	±2.3	16.8	±4.1	14.5	±2.3	1.1	2.1	3.7	1.8	3.2	3.7

SUPPLEMENTARY INFORMATION (S.I.)

Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF

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Experimental Section

Reagents

The illicit drugs, pharmaceuticals and their metabolites reference substances selected for target analysis were cocaine, cocaethylene, benzoylecgonine, amphetamine, methamphetamine, 3,4-methylene-dioxymethamphetamine (MDA), 3,4-methylenedioxyethylamphetamine (MDMA or ecstasy),para-hydroxymethamphetamine, para-methoxyamphetamine (PMA), para-methoxy-N-methylamphetamine (PMMA), ketamine, dehydronorketamine, norketamine, methoxetamine, pseudoephedrine, cathinone, methcathinone (ephedrone), methylone, 4´-methyl-N-ethylcathinone (4-MEC), mephedrone (4-MMC),methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), morphine, 6-monoacetylmorphine (6-MAM), fentanyl, buprenorphine, AH-7921, hydroxycotinine, atenolol, salbutamol, gabapentin, paracetamol, metoprolol, methylphenidate, propranolol, citalopram, atomoxetine, carbamazepine, midazolam, nitrazepam, clonazepam, lorazepam, oxazepam, alprazolam, flunitrazepam, etizolam, phenazepam, diazepam, diclofenac, etizolam, carbamazepine, Δ-9-tetrahydrocannabidiol (THC), 11-hydroxy-Δ-9-tetrahydrocannabidiol (OH-THC), 11-nor-9-carboxy-Δ-9-tetrahydrocannabidiol (THC-COOH), which were obtained from Nerliens Meszansky (Oslo, Norway) as solutions in methanol (MeOH) or acetonitrile (ACN) at concentrations of 1 mg mL-1. Standard solutions of each compound were made at 100 ng mL-1 in methanol and then diluted into a final mix solution to a concentration of 1 ng mL-1

Synthetic cannabinoids and their selected biomarkers, JWH-073, XLR-11, UR-144, AM-2201, JWH-018, MAM-2201, JWH-122, 5-3-1-Naphthoyl-1H-indol-1-yl-pentanoic acid (JWH-018 N-pentanoic acid), 1-5-hydroxypentyl-1H-indol-3-ylnaphthalen-1-yl-methanone (JWH 018 N-5-hydroxypentyl), 4-3-1-naphthoyl-1H-indol-1-yl-butanoic acid (JWH-073 N-butanoic acid), 1-4-hydroxybutyl-1H-indol-3- ylnaphthalen-1-yl-methanone (JWH-073 N-4-hydroxybutyl), 1-5-hydroxypentyl-1H-indol-3-yl4-methylnaphthalen-1-yl-methanone (JWH-122 N-5-hydroxypentyl), 1-5-fluoro-4-hydroxypentyl-1H-indol-3-ylnaphthalen-1-ylmethanone (AM-2201 N-4-hydroxypentyl), 1-5-hydroxypentyl-1H-indol-3-yl4-methoxyphenyl-methanone (RCS-4 N-5-hydroxypentyl) were obtained from Chiron (Trondheim, Norway) in solutions of 1 mL at 50 μg mL-1 in MeOH or ACN.

Benzo Fury, 1-benzofuran-6-ylpropan-2-amine (6-APB) or 1-benzofuran-5-ylpropane-2-amine (5-APB), and 2NE1/APICA, N-adamantyl-1-pentylindole-3-carboxamide were purchased through online website.

Deuterated standards were purchased from Nerliens Meszansky as solutions of 100 ng mL-1 in MeOH or ACN and were used as surrogate isotope labelled internal standards (ILIS) for quantification: atenolol-d7, atomoxetine-d7, diclofenac-d4, gabapentin-d10, metoprolol-d7, pregabalin-d6, benzoylecgonine-d3, cocaine-d3, ketamine-d4, norketamine-d4, mephedrone-d3, methcathinone-d3 (ephedrone-d3), methylone-d3, pseudoephedrine-d3, buprenorphine-d4, diazepam-d5, oxazepam-d5, fentanyl-d5, methadone-d3, amphetamine-d5, MDA-d5, MDMA-d5, methamphetamine-d5, THC-OH-d3, THC-COOH-d3. Final standard mix solution was made in methanol at 1 ng mL-1. Finally all the standard solutions were stored in amber glass bottles at -20 °C.

The ultrapure water was obtained by purifying demineralized water in an Elga Maxima Ultrapure Water purification system (Elga, Lane End, UK). Ammonium formate (for mas spectroscopy, ≥99.0%), HPLC-grade formic acid (eluent additive for LC-MS) and UHPLC-grade water, MeOH and ACN were acquired from Sigma-Aldrich, Fluka for HPLC (Oslo, Norway)

Sewage-based samples

Influent samples were collected from two wastewater treatment plants (WWTWs); VEAS WWTW in Oslo and Ladehammeren and Høvringen WWTWs in Trondheim Norway.

Nine influent wastewater composite samples were collected during three consecutive weekends from VEAS sewage treatment plant, Oslo Norway, in February 2014. An ISCO Avalanche Portable Refrigerated Sampler (Lincoln, NE, USA) was used to collect the composite samples (50 mL, time proportional) every 30 minutes for the duration of the sampling period. Maximum storage time for the daily composite samples was 3 days at 0.5ºC in polypropylene bottles. The composite samples were transported every Monday in cooler bags and stored at -20 ºC until analysis to minimize the degradation of the analytes. The catchment area of VEAS is comprised of 63% of the Oslo population, around 600,000 inhabitants. The total length of the tunnel is 42.3 km and the mean residence time in the sewer system is 5 hours (VEAS annual report 13'). The mean influent flow rate for each weekend was 600,820, 775,327, and 410,970 m3/day, which is higher that the annual mean (273,196 m3 day-1 in 2013) and was due to increased precipitation during the sampling campaign.

Six influent 24-hour composite wastewater samples were collected during February, March and April of 2014 in two different sewage treatment plant located in Trondheim (Norway). Ladehammeren and Høvringen WWTPs treat the sewage from the city with an estimated population of 180,000 inhabitants. Samples were received frozen and analysed upon reception in the laboratory. Mean influent flow rates are described in the results section.

The robustness of this work was also examined with the analysis of different sewage-based samples. The "Pharmaceuticals" version of the POCIS was made up of Oasis HLB sandwiched between polyethersulphone membranes as described in literature[1]. POCIS were deployed in the sedimentation overflow channel at VEAS, the Oslo WWTP, for two weeks. The study and collection of the pooled urine samples was conducted in Norway during the course of three different music festivals. Portable toilets were available to be used by the festival participants anonymously and no data on the number of users was collected.

Extraction procedure

The HLB extraction disks were conditioned by washing and rinsing with methanol (10 mL) and water (10 mL). Samples were automatically loaded and filtered (Fast Flow Pre-Filters, Horizon Technology) onto the disks at a flow rate of 100 mL min-1, and then the disks were washed 4 times with 5% methanol in water and dried under vacuum for 10 minutes.

Then the samples were loaded directly onto the disk from the sample bottle and prior elution, the disk is air-dried under vacuum providing a shorter operational time. The analytes were finally eluted into a silanised glass vial with 2 cycles of 5% ammonium hydroxide in methanol and 2 cycles of 5% acetic acid in methanol. The total program time was approximately 25 minutes. In general this system is more simple, reproducible and cost-effective than the SPE cartridges.

The final extracts were evaporated to around 100 μL under a gentle nitrogen stream at 35 ºC and reconstructed in 400 μL of 25% methanol aqueous solution. An aliquot was centrifuged and then 5 μL were injected into the UHPLC-QTOF. The general procedure is also described in the Table S-1.

The "Pharmaceuticals" version of the POCIS were deployed in the wastewater treatment plant and subsequently the sorbent was removed and introduced in an empty SPE cartridge with Mili-Q water, washed twice with 6 mL of 5 % methanol in water and finally eluted with 6 mL of 0.5% ammonium hydroxide in methanol and 6 mL of 0.5% acetic in methanol. The eluent was evaporated under a stream of nitrogen and diluted to 1.5 mL with 13% methanol in water. 100 ng of the ILIS mix was added during the washing step.

The pooled urine samples were firstly homogenized and then centrifuged during 20 minutes at 2500 rpm. 5 mL of the supernatant was collected, spiked with 100 ng of the ILIS mix and introduced into the HLB cartridge. Washed with 2x6 mL 5 % methanol in water, the elution was finally done with 6 mL of 0.5% ammonium hydroxide in methanol and 6 mL of 0.5% acetic in methanol. The eluent was evaporated under a stream of nitrogen and diluted to 1 mL with 13% methanol in water.

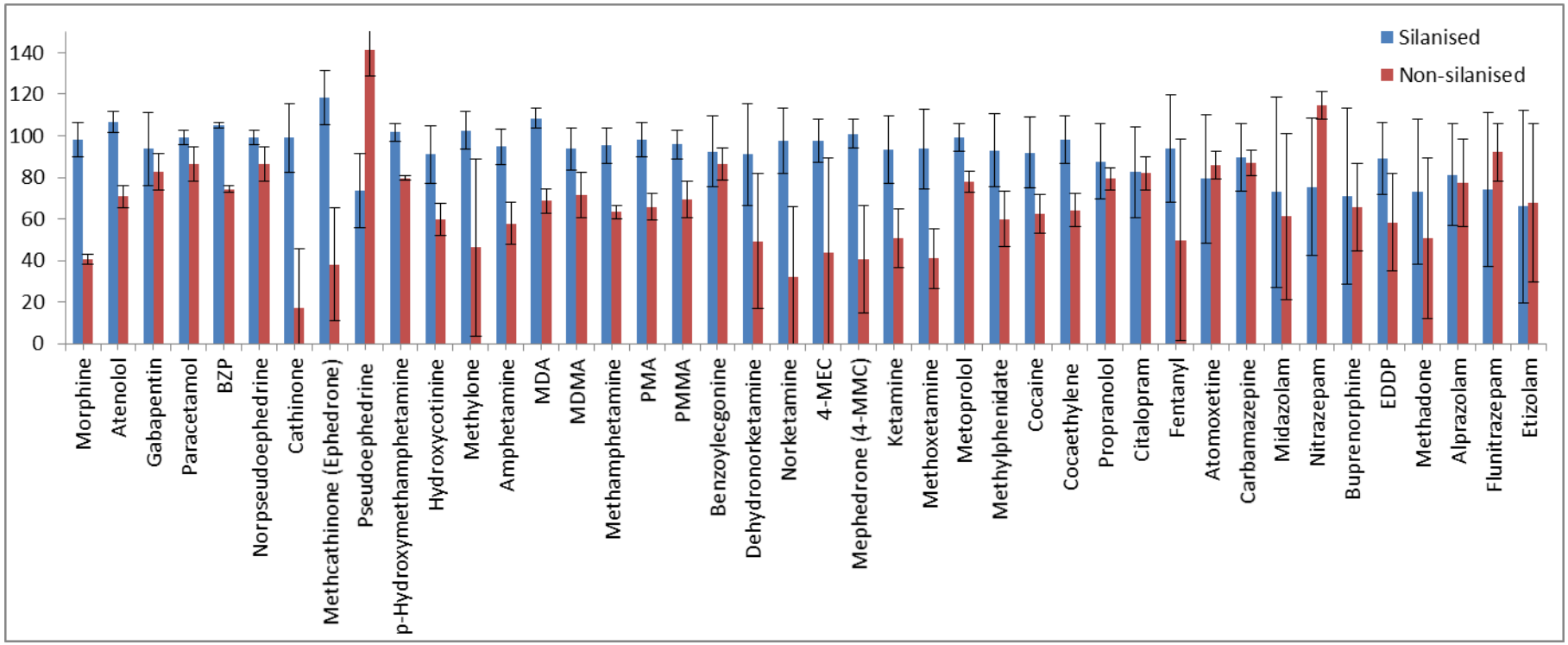

Figure S-1
Influence of silanisation on the recovery (%) of the studied compounds during the evaporation in the SPE extract vials. Recovery of 100 mL of MiliQ water spiked with 100 ng of the standard mix solution using HLB cartridges (n=3)

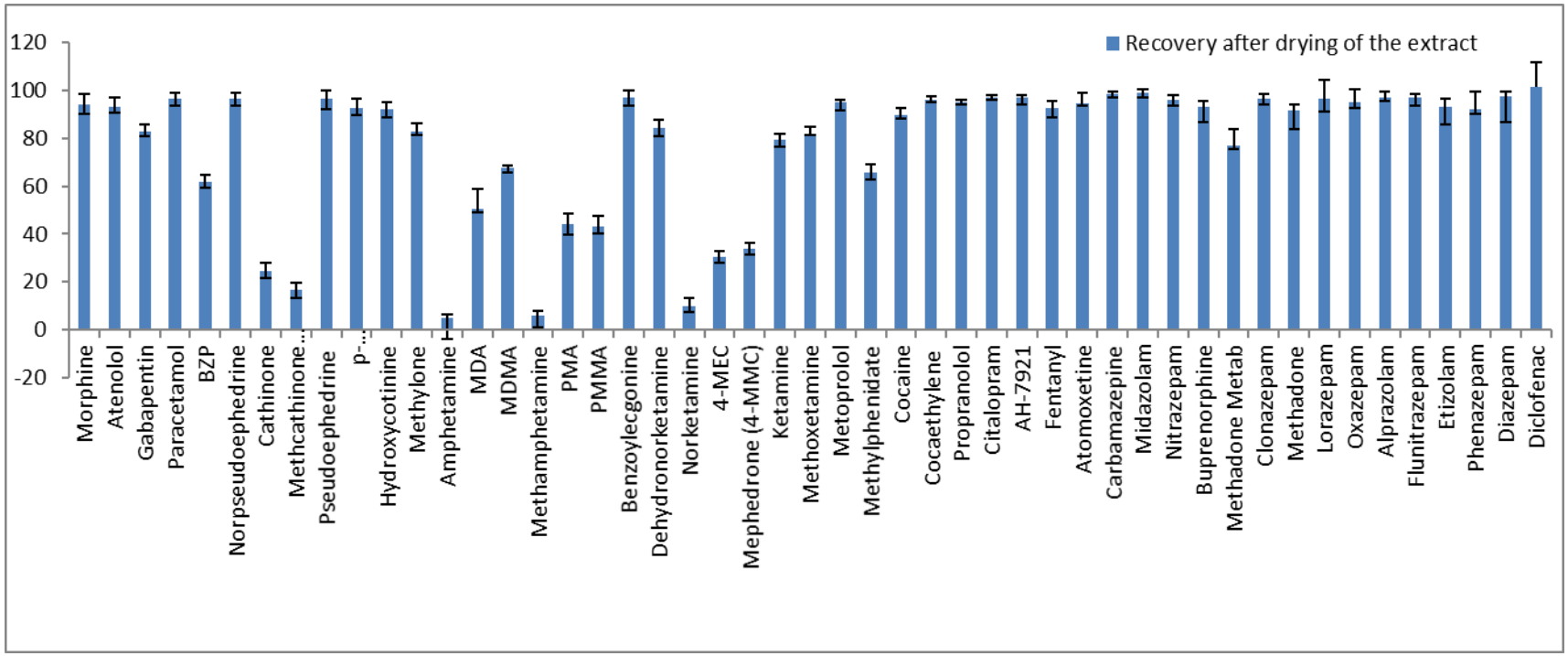

Figure S-2
Recovery of the studied compounds after the evaporation to dryness at 50 °C of a 5 mL methanol solution with 100 ng of the mix standard solution (n=3)

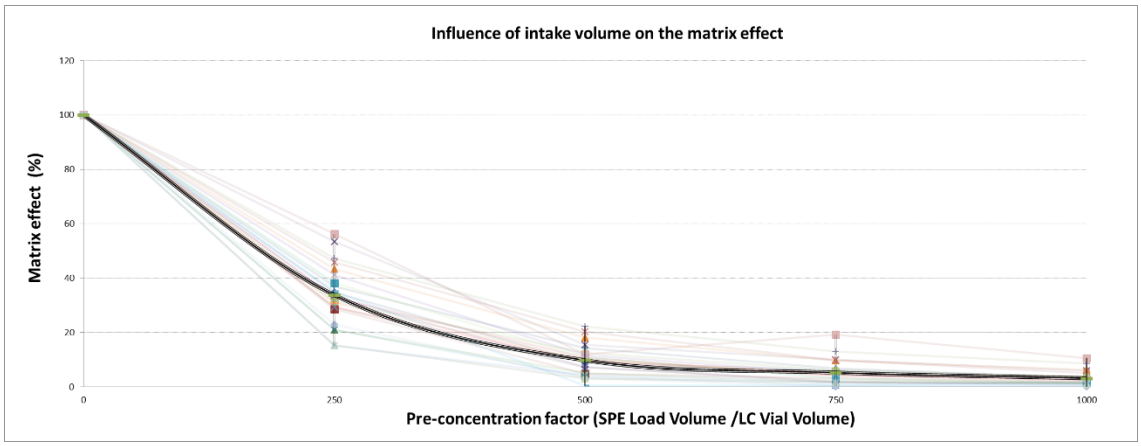

Figure S-3
Effect of the loading volume on the matrix effect. Matrix suppression average for 25 ILIS spiked into the extracts of 3 different wastewater samples. The loading volumes were 100, 200, 300, and 400 mL and the final extract was reconstituted volume of 400 µL

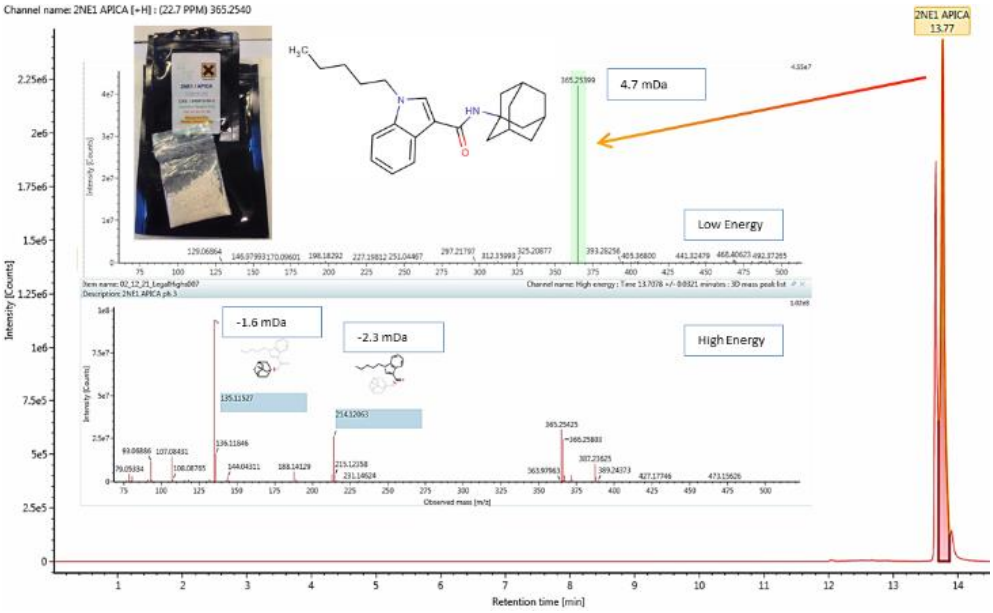

Figure S-1
Detection and identification of N-adamantyl-1-pentylindole-3-carboxamide in a sample purchased on internet under the name of "2NE1/APICA". Extracted ion chromatogram of the 2NE1/APICA powder sample and the spectra in low and high energy time-of-flight. Possible fragment structures assigned manually by UNIFI

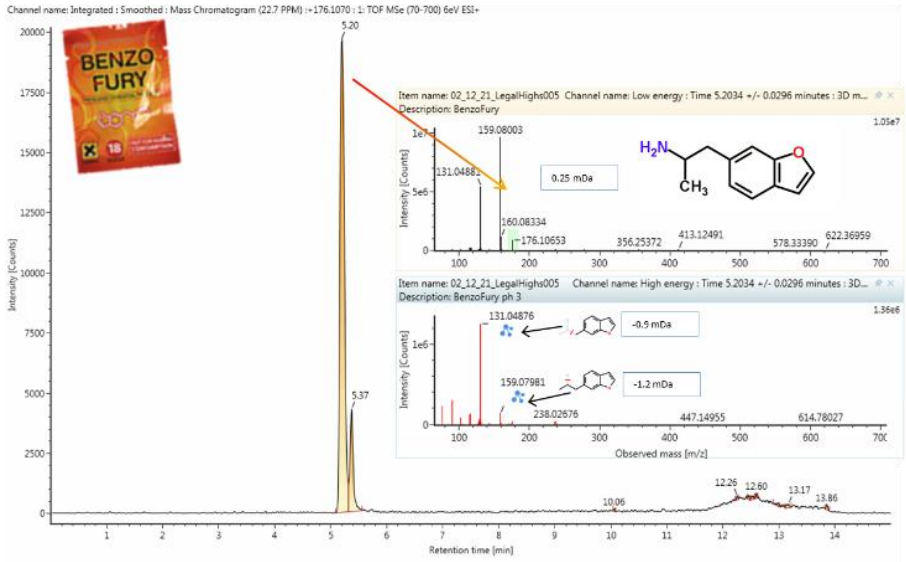

Figure S-2
Detection and identification of 6-(2-aminopropyl)benzofuran (6-APB)/ 5-APB (5-(2-aminopropyl)benzofuran (5-APB) in a sample purchased on internet under the name of "Benzo Fury". Extracted ion chromatogram of the Benzo Fury tablet sample and the spectra in low and high energy time-of-flight. Possible fragment structures assigned manually by UNIFI

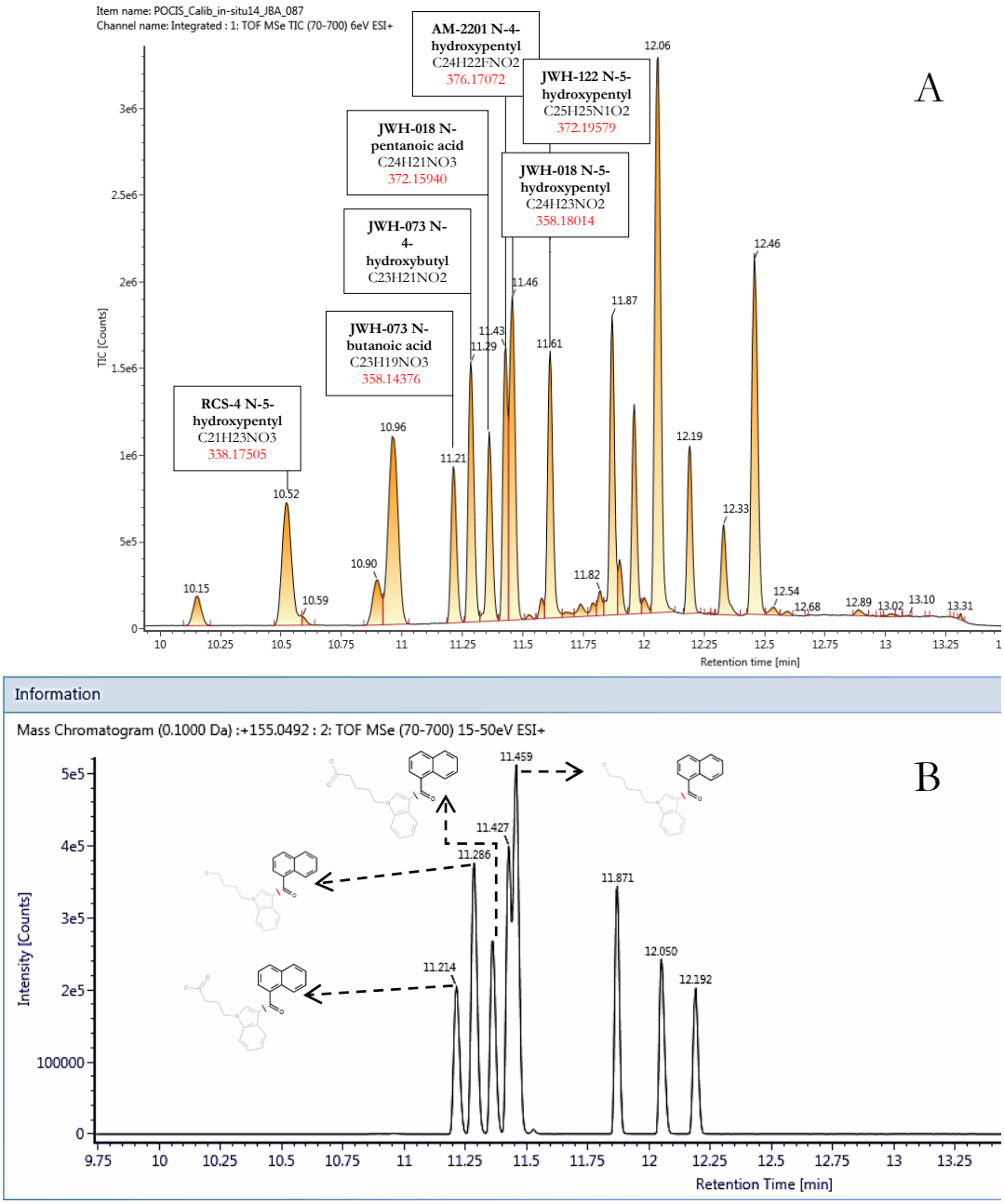

Figure S-6
Total ion chromatogram for the 14 synthetic cannabinoids of a standard mix at 250 ng/mL with the description of the main metabolites A). Common fragment (155.0492) chromatogram from four synthetic cannabinoids and their four metabolites B)

Table S-1
SPE-DEX procedure

Step	Solvent	Soak time	Dry time
Preconditioning	Methanol (2 times)	20 s	10 s
	Water	20 s	10 s
	Water	20 s	0 s
Sample loading	-	-	-
Washing	5% methanol/water(4 times)	10 s	30 s
Disk drying	-	-	10 min
Eluting	5% ammonium hydroxide in methanol	30 s	30 s
	5% ammonium hydroxide in methanol	30 s	2 min
	5% acetic acid in methanol	30 s	30 s
	5% acetic acid in methanol	30 s	2 min

Table S- 2
Experimental parameters used for the validation and quantification of the target compounds: chromatographic retention times, exact mass of the protonated target compounds, validated linear range, correlation coefficient, mass error and the results of the instrumental performance

Compound	t_R	*Precursor ion*	*Linear range*	*Linearity* R^2	*Mass Error*[a]	Accuracy (%)				*Precision (RSD%)*			
						Intra-day		Inter-day		*Intra-day*		Inter-day	
		[M + H]+	*(ng mL^{-1})*		*(mDa/ppm)*	*Low*	*High*	*Low*	*High*	*Low*	*High*	*Low*	*High*
Morphine	1.17	286.14376	0,5-100	0.995	-0.08/-0.29	94	101	94	101	1	1	3	2
Hydroxycotinine	1.17	193.09714	0,5-400	0.999	0.09/046	92	97	88	98	3	2	5	5
Atenolol	1.26	267.1703	0,5-200	1.000	-0.05/-0.2	108	99	108	99	3	0	3	0
Salbutamol	1.28	240.1594	1-100	0.995	-0.17/-0.67	108	100	107	99	2	1	5	2
p-Hydroxymethamphetamine	1.31	166.12263	2-100	0.998	-0.17/-0.99	96	102	99	101	7	2	7	2
Paracetamol	1.62	152.07059	2-400	0.998	0/-0.02	98	101	104	101	3	2	9	5
Cathinone	1.75	150.09133	5-400	0.991	-0.08/-0.52	93	104	92	104	3	2	3	1
Gabapentin	1.8	172.13319	5-200	0.995	-0.04/-0.26	110	102	108	99	3	1	2	6
Pseudoephedrine	1.96	166.12263	5-400	0.998	-0.31/-2.07	96	99	94	99	9	1	8	4
Methcathinone (Ephedrone)	1.98	164.10698	1-400	0.998	-0.35/-2.49	93	100	96	100	1	0	3	0
Methylone	2.16	208.09681	0,25-400	0.999	0.03/0.12	110	100	109	100	4	0	3	0
6-MAM	2.29	328.15432	0,5-400	0.998	-0.01/-0.05	95	102	98	101	3	1	4	0
Amphetamine	2.41	136.11207	5-400	0.993	-0.15/-1.31	88	104	98	104	5	1	12	1
MDA	2.47	180.10189	10-200	0.994	0.04/0.29	109	104	108	103	6	4	6	4
PMA	2.53	166.12263	2-400	0.999	-0.42/-2.59	110	100	107	100	2	1	5	1
Methamphetamine	2.72	150.12771	2-200	0.999	-0.16/-1.10	105	101	104	100	4	1	5	1

MDMA	2.75	194.11754	2-400	0.999	-0.03/-0.16	100	100	100	100	4	1	3	1
Dehydronorketamine	2.89	222.06801	1-400	0.998	0.22/0.98	106	99	108	97	7	0	7	2
PMMA	2.98	180.13828	2-400	1.000	-0.42/-2.59	101	100	100	100	3	0	3	1
Benzoylecgonine	3.07	290.13867	0,25-400	0.999	0.01/0.07	94	101	95	101	2	0	1	0
Mephedrone (4-MMC)	3.19	178.12263	2-400	0.999	-0.14/-0.86	107	99	105	99	8	0	8	0
Norketamine	3.21	224.08366	5-200	0.999	0.12/0.58	109	102	104	101	4	1	7	6
Ketamine	3.45	238.09931	0,5-400	1.000	-0.35/-2.49	98	99	97	99	3	1	4	1
4-MEC	3.7	192.13828	0,5-400	1.000	-0.09/-0.48	97	99	98	99	2	1	2	1
Methoxetamine	4.12	248.16449	0,25-400	1.000	-0.06/-0.21	105	99	105	99	7	0	5	0
Metoprolol	4.19	268.1907	0,25-400	0.999	-0.16/-0.61	102	100	101	100	1	1	2	1
Methylphenidate	4.32	234.14884	0,25-400	1.000	0/-0.05	98	100	94	100	3	1	5	1
Cocaine	4.83	304.15432	0,25-200	1.000	0/0.03	97	101	97	101	2	0	1	0
Cocaethylene	5.87	318.16997	0,25-400	1.000	0.04/-0.13	96	102	96	101	1	1	1	1
Propranolol	6.28	260.16449	0,25-200	0.998	-0.04/-0.13	105	99	105	94	2	1	2	6
Fentanyl	6.63	337.22742	0,25-400	1.000	-0.24/-0.69	107	99	105	99	4	0	5	1
AH-7921	7.02	329.11818	0,25-400	1.000	-0.1/-0.33	110	99	113	100	4	0	5	2
Citalopram	7.07	325.17105	0,25-200	0.997	-0.14/-0.42	100	100	100	100	4	1	3	1
Midazolam	7.14	326.08547	0,25-400	0.999	-0.03/-0.1	106	99	108	97	7	0	7	2
Buprenorphine	7.38	468.31081	0,25-400	1.000	0.11/0.25	101	100	100	100	3	0	3	1
Carbamazepine	7.54	237.10223	0,25-200	1.000	0.19/0.75	94	101	95	101	2	0	1	0
EDDP	7.75	278.19031	0,25-400	0.999	-0.1/-0.33	107	99	105	99	8	0	8	0
Atomoxetine	7.81	256.16958	1-200	0.999	-0.02/-0.07	109	102	104	101	4	1	7	6

Nitrazepam	8.16	282.08731	1-400	0.998	-0.09/-0.33	98	99	97	99	3	1	4	1
Oxazepam	8.22	287.05817	2-400	0.997	-0.51/-1.78	97	99	98	99	2	1	2	1
Clonazepam	8.5	316.04833	1-400	0.998	-0.35/-1.09	105	99	105	99	7	0	5	0
Lorazepam	8.6	321.0192	5-400	0.994	-0.22/-0.69	102	100	101	100	1	1	2	1
Alprazolam	8.83	309.09014	0,25-200	0.996	-0.04/-0.15	98	100	94	100	3	1	5	1
Methadone	8.89	310.21652	0,25-400	0.999	0.39/1.24	97	101	97	101	2	0	1	0
Flunitrazepam	9.19	314.09353	1-400	1.000	-0.07/-0.18	96	102	96	101	1	1	1	1
Etizolam	9.53	343.07786	0,25-200	1.000	-0.08/-0.26	105	99	105	94	2	1	2	6
Phenazepam	10.1	348.97382	2-400	0.998	-0.28/-0.82	107	99	105	99	4	0	5	1
Diazepam	10.91	285.07891	0,25-200	1.000	0.05/0.19	110	99	113	100	4	0	5	2
Diclofenac	11.4	296.02395	5-400	0.997	-0.02/-0.08	100	100	100	100	4	1	3	1
THC-OH	11.83	331.22675	5-400	0.995	-0.17/-0.56	106	99	108	97	7	0	7	2
THC-COOH	11.85	345.20602	5-400	0.992	0.17/0.5	101	100	100	100	3	0	3	1

Table S-3
Method validation in influent wastewater (n=6)

Compound	*Internal Standard*	*Matrix Suppression*	*Recovery* 100 ng L^{-1}	CV(%)	600 ng L^{-1}	CV(%)	*Instrumental LOQ (ng mL-1)*	*Theoretical LOQ (ng L^{-1})*		*Information*
Morphine	Atenolol-d_7	38	73	9	68	10	0.40	4.2		
Hydroxycotinine	Atenolol-d_7	38	120	19	88	17	0.57	5.9	*	high concentrations in "blank"
Atenolol	Atenolol-d_7	38	92	2	91	12	0.35	3.7		
Salbutamol	Atenolol-d_7	38	88	7	90	8	0.81	8.5		
~~p-Hydroxymethamphetamine~~	Atenolol-d_7	38	-	-	-	-	1.34	14.0	*	high matrix suppression
Paracetamol	Atenolol-d_7	38	-	-	-	-	1.28	13.4	*	high concentration in "blank"
Cathinone	Methcathinone-d_3	34	~LOQ	~LOQ	51	2	5.69	67.5		
~~Gabapentin~~	Gabapentin-d_{10}	30	-	-	-	-	3.79	51.2	*	high matrix suppression
Pseudoephedrine	Methcathinone-d_3	34	101	5	118	5	3.10	36.7		
Methcathinone (Ephedrone)	Methcathinone-d_3	34	85	7	94	1	0.85	10.0		
Methylone	Methylone-d_3	37	77	2	98	3	0.04	0.4		
6-MAM	Methylone-d_3	37	72	5	81	4	0.30	3.2		
Amphetamine	Amphetamine-d_5	14	77	9	90	13	3.06	85.6		
~~MDA~~	MDA-d_5	26	-	-	-	-	11.95	187.0	*	high matrix suppression
PMA	MDMA-d_5	26	57	5	77	8	1.03	16.1		
MDMA	MDMA-d_5	26	69	4	69	3	1.53	24.0		
Methamphetamine	Methamphetamine-d_5	11	77	10	83	5	1.51	54.1		

Dehydronorketamine	Norketamine-d_4	33	48	2	54	4	0.65	8.0
PMMA	MDMA-d_5	26	74	8	79	2	0.90	14.0
Benzoylecgonine	Benzoylecgonine-d_3	51	102	9	107	7	0.12	0.9
Mephedrone (4-MMC)	Mephedrone-d_3	18	61	2	74	6	1.16	26.3
Norketamine	Norketamine-d_4	33	76	5	76	11	2.89	35.5
Ketamine	Ketamine-d_4	41	80	4	100	6	0.26	2.5
4-MEC	Ketamine-d_4	41	73	4	91	5	0.28	2.7
Methoxetamine	Metoprolol-d_7	40	32	7	41	3	0.19	1.9
Metoprolol	Metoprolol-d_7	40	84	12	95	6	0.11	1.1
Methylphenidate	Metoprolol-d_7	40	47	9	51	9	0.15	1.5
Cocaine	Cocaine-d_3	52	93	11	103	7	0.07	0.6
Cocaethylene	Cocaine-d_3	52	90	9	101	6	0.11	0.9
Propranolol	Fentanyl-d_5	33	102	18	119	11	0.06	0.8
AH-7921	Fentanyl-d_5	33	97	5	108	12	0.20	2.4
Citalopram	Buprenorphine-d_4	24	72	14	84	10	0.09	1.5
Fentanyl	Fentanyl-d_5	33	90	14	112	3	0.14	1.7
Midazolam	Buprenorphine-d_4	24	98	3	119	6	0.09	1.5
Buprenorphine	Buprenorphine-d_4	24	86	7	115	4	0.15	2.5
Carbamazepine	Buprenorphine-d_4	24	118	6	130	6	0.14	2.3
EDDP	Atomoxetine-d_7	23	77	16	103	11	0.11	2.0
Atomoxetine	Atomoxetine-d_7	23	71	1	62	10	0.62	10.8
Nitrazepam	Oxazepam-d_5	78	76	3	93	5	0.74	3.8

Clonazepam	Oxazepam-d_5	78	57	1	46	1	1.14	5.9		
Oxazepam	Oxazepam-d_5	78	113	15	104	14	2.23	11.5		
Lorazepam	Oxazepam-d_5	78	86	3	87	2	2.31	11.9		
Alprazolam	Oxazepam-d_5	78	75	15	100	14	0.16	0.8		
Methadone	Methadone-d_3	41	97	8	96	5	0.04	0.4		
Flunitrazepam	Diazepam-d_5	19	73	7	100	15	0.71	15.2		
Etizolam	Diazepam-d_5	19	66	13	84	10	0.09	2.0		
Phenazepam	Diazepam-d_5	19	104	9	118	9	1.50	32.2		
Diazepam	Diazepam-d_5	19	125	7	110	14	0.10	2.2		
~~Diclofenac~~	Diazepam-d_5	19	-	-	-	-	2.94	62.9	*	high matrix suppression
~~THC-OH~~	THC-OH-d_3	19	-	-	-	-	2.41	50.3	*	high matrix suppression
~~THC-COOH~~	THC-COOH-d_3	17	-	-	-	-	3.22	76.7	*	high matrix suppression

Table S-4
Stability of the spiked analytes (1000 ng L^{-1}) in influent WW (n=3) at pH 7. Difference (%)/ ± SD

	Peak Area Difference (%)± SD					
Compound	***5 h***		***8 h***		***120 h***	
Morphine	-24	±20	-5	±17	83	±12
Hydroxycotinine	22	±5	82	±1	54	±4
Atenolol	-9	±5	-10	±4	-20	±2
Salbutamol	9	±10	1	±9	-3	±8
p-Hydroxymethamphetamine	7	±14	-1	±9	70	±22
Paracetamol	-1	±14	-8	±8	-88	±8
Gabapentin	-9	±25	-28	±8	-32	±11
Pseudoephedrine	9	±10	13	±15	0	±11
Methcathinone (Ephedrone)	-4	±18	-8	±22	-33	±11
Methylone	6	±62	-27	±21	-53	±12
6-MAM	-14	±7	-16	±8	-21	±7
Amphetamine	14	±3	23	±6	33	±7
MDA	15	±11	-2	±18	-96	±18
PMA	3	±20	16	±7	10	±7
MDMA	29	±36	6	±6	12	±6
Methamphetamine	-10	±8	-15	±33	23	±29
Dehydronorketamine	-11	±11	-7	±20	-13	±18
PMMA	-4	±33	17	±9	6	±10
Benzoylecgonine	19	±8	30	±7	56	±8
Mephedrone (4-MMC)	-1	±1	3	±1	7	±1
Norketamine	-12	±6	-24	±17	-25	±20
Ketamine	4	±27	-13	±8	-25	±5
4-MEC	5	±16	-2	±18	-32	±12
Methoxetamine	-4	±4	-6	±10	13	±0
Metoprolol	-6	±3	-9	±3	-5	±3
Methylphenidate	-10	±11	-32	±4	-88	±11
Cocaine	-9	±6	-23	±8	-75	±3
Cocaethylene	-1	±5	-6	±5	-42	±4
Propranolol	8	±6	6	±5	-27	±6
AH-7921	18	±15	9	±5	-26	±11
Citalopram	13	±9	14	±6	-23	±7
Fentanyl	9	±13	10	±2	-25	±0
Midazolam	-3	±7	5	±10	-54	±8
Buprenorphine	18	±12	23	±10	-41	±14
Carbamazepine	4	±6	4	±6	-11	±5
EDDP	45	±4	82	±5	77	±6
Atomoxetine	20	±4	22	±1	-60	±7
Nitrazepam	-14	±12	-8	±9	-79	±9
Clonazepam	-2	±11	-3	±10	-65	±9
Oxazepam	-7	±8	-1	±8	-36	±8
Lorazepam	-1	±10	-3	±7	-46	±11
Alprazolam	-1	±6	3	±7	-23	±8
Methadone	3	±6	7	±8	-41	±7
Flunitrazepam	0	±5	0	±7	-64	±4
Etizolam	-1	±5	2	±10	-41	±7
Phenazepam	2	±8	3	±15	-70	±9
Diazepam	-5	±7	-8	±10	-59	±7

Table S-5
Concentration values (ng/POCIS) for the most commonly detected drugs in the passive samplers

Analyte	*Date of collection and concentration values (ng/POCIS)*									
	29.08.2013	***16.09.2013***	***30.09.2013***	***15.10.2013***	***29.10.2013***	***15.11.2013***	***02.12.2013***	***18.18.2013***	***03.01.2014***	***21.01.2014***
Morphine	34.9	39.1	48.2	22.3	22.9	18.6	25.1	22.1	16.2	30.8
Hydroxycotinine	18.7	14.6	17.1	44.7	28.4	40.9	24.5	36.9	27.8	31.7
Atenolol	68.0	68.4	89.5	36.4	44.9	45.7	69.8	44.0	26.3	58.3
Paracetamol	718.6	521.1	940.7	778.3	779.2	699.9	1086.3	1091.3	360.5	1055.9
MDMA	33.1	33.9	48.4	25.3	25.5	4.8	49.4	32.9	20.8	26.9
Benzoylecgonine	78.0	77.8	85.6	43.1	52.7	46.1	86.0	58.9	39.4	58.9
Methoxetamine	3.3	3.5	3.8	1.5	1.9	2.0	2.6	1.4	1.2	1.8
Metoprolol	448.5	518.4	738.6	384.7	358.3	408.5	541.5	436.6	194.7	370.0
Cocaine	106.1	110.4	133.9	69.5	68.2	72.9	121.8	85.4	38.3	68.8
Propranolol	38.3	54.5	31.2	32.5	31.5	42.9	41.5	40.2	14.3	24.6
Citalopram	93.8	119.1	87.6	72.7	70.2	118.0	132.8	103.7	27.2	74.0
Carbamazepine	244.3	399.5	440.1	226.0	226.3	205.8	267.1	220.0	98.8	177.6
EDDP	97.1	169.4	139.4	103.9	81.9	91.9	114.9	78.7	20.4	74.5
Methadone	15.1	17.2	18.9	15.8	13.1	15.2	16.9	15.1	7.0	11.3
Oxazepam	696.9	927.5	744.8	692.4	525.6	675.3	754.7	745.0	187.1	421.1
Diclofenac	134.2	144.8	109.9	92.2	149.7	103.5	133.9	125.3	47.4	102.8

Table S-6
Concentration (ng/L) values of target compounds in pooled urine samples (5 mL) from anonymous Norwegian music festivals

Compound	Festival 1	Festival 2	Festival 3
Atenolol	-	900	-
Morphine	-	-	440
Paracetamol*	3.6	42.2	1320.2
Gabapentin	-	5960	-
Amphetamine	5840	-	20900
Methamphetamine	1360	820	1780
MDMA*	-	-	60.6
MDA	-	-	4340
Cocaine	-	480	520
Benzoylecgonine	1340	78820	14680
Cocaethylene	-		
Citalopram	-	-	2360
Propranolol	540	-	720
Carbamazepine	3520	7860	-
Oxazepam	340	1100	-
Methylphenidate	-	-	
Methadone	240	-	-
THC-COOH	1020		7080

*(µg/L)

Table S-7. Occurrence of the identified suspect candidates in the pooled urine samples by UHPLC-QTOF using the suspect database.

Compound	Number of Identified LE Ions	Retention Time	Elemental Composition	m/z
1-Benzylpiperazine (BZP)	2	1.23	C11H16N2	176.13134
Hydroxy-metoprolol	3	1.43	C15H25NO4	283.17834
Caffeine	3	2.08	C8H10N4O2	194.08036
Nadolol	3	2.27	C17H27NO4	309.19399
Metoclopramide	4	3.02	C14H22ClN3O2	299.14004
Lidocaine	2	3.19	C14H22N2O	234.1732
O-demethyl-venlafaxine	3	3.2	C16H25NO2	263.18851
Remifentanil	3	4.37	C20H28N2O5	376.1998
Disopramide	3	4.56	C21H29N3O	339.23104
Zolpidem	4	5.19	C19H21N3O	307.16845
Venlafaxine	3	5.29	C17H27NO2	277.20416
Acetildenafil	5	5.37	C25H34N6O3	466.26922
Enalapril	2	5.49	C20H28N2O5	376.1998
Mepyramine	2	5.52	C17H23N3O	285.1841
Sildenafil	2	6.19	C22H30N6O4S	474.2049
Benzocaine	2	6.5	C9H11NO2	165.07897
Imipramine	2	7.67	C19H24N2	280.19393
Cetirizine	2	8.28	C21H25ClN2O3	388.15535
Trimipramine	2	8.44	C20H26N2	294.20958
Irbesartan	2	9.08	C25H28N6O	428.23244
Irbesartan	2	9.28	C25H28N6O	428.23244
Cinnarizine	2	10.48	C26H28N2	368.22523
Fluocinonide	2	11.17	C26H32F2O7	494.21159
Orlistat	4	13.24	C29H53NO5	495.39234

(1) Harman, C.; Reid, M.; Thomas, K. V. *Environ Sci Technol* **2011**, *45*, 5676-5682.